DESCRIPTION

CANCER GENE THERAPEUTIC DRUG

Technical field

This disclosure relates to a cancer gene therapeutic drug, and a therapeutic method of using a cancer gene as a therapeutic drug.

Background art

Recently, cancer gene therapy has received attention as a cancer therapy. A variety of gene therapies have been proposed for testing and some clinical trials have been conducted to test their effect. Among them, a clinical trial was performed by Freeman (Freeman, SM, et al., The treatment of ovarian cancer with a gene modified cancer vaccine: a phase I study, Hum Gene Ther., 1995 July 6(7):927-39) to test a cancer gene therapy using carrier cells. This cancer gene therapy used an ovarian cancer cell (PA-1) with a HSV-tk gene from a retrovirus as the carrier cell. The clinical trial tested it for ovarian cancer therapy, as well as, malignant mesothelioma therapy (see Paul Schwarzenberger, P., et al., Clinical Protocol The Treatment of Malignant Mesothelioma with a Gene Modified Cancer Cell Line: A Phase I Study, Human Gene Therapy. November 20, 1998, 9(17): 2641-2649 Human Gene Therapy, 9, 2641 - 2649, 1998). Culver (Culver, KW, et al., In vivo gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors, *Science* 12 June 1992 256: 1550-1552) used the mouse cell NIH-3T3 as the carrier cell and conducted a clinical trial to test it against a cerebral tumor. However, its application for human cancer therapy requires human derived cells as the carrier cells.

A gene therapy using the ovarian cancer cell PA-1 as the carrier cell was also carried out (Coukos, G., et al., Use of Carrier Cells to Deliver a Replication-selective Herpes Simplex Virus-1 Mutant for the Intraperitoneal Therapy of Epithelial Ovarian Cancer, Clin. Cancer Res., 1999 5: 1523-1537). This gene therapy uses an oncolytic virus, which specifically proliferates in tumor cells. The virus is infected into the carrier cells (producer cells) and then the infected carrier cells are administered into the tumor site. Herpes simplex 1 (HSV-1) is used as the oncolytic virus. In an animal experiment, the infected carrier cells are injected intraperitoneally into a nude mouse model with ovarian cancer transferred to the peritoneal cavity (International

Publication No. 99/45783 (pamphlet) and International Publication No. 01/23004 (pamphlet)).

The ovarian cancer cells PA-1 show the ability to highly proliferate and be easily manipulated, but they have a drawback of being fragile with a small cytoplasm. Therefore, introduction of the HSV-tk gene by retrovirus gives little expression of the HSV-tk gene in the tumor site and no satisfactory antitumor effect was obtained against ovarian cancer or malignant mesothelioma.

The use of PA-1 as the carrier cell in the cancer gene therapy with the oncolytic virus HSV-1 showed no significant antitumor effect in comparison to that of a therapy with only the oncolytic virus HSV-1. Repeated administrations of this cancer gene therapy with a virus cannot be conducted because of the production of neutralizing antibodies in the blood against the cells. Using PA-1 cells results in little production of the virus due to its fragility. The cell disruption before infection to the target tumor cells by cell to cell interaction, and inactivation of the virus by neutralizing antibodies may produce no significant antitumor effect.

Furthermore, the patient's own cancer cells or fibroblasts could be used as the carrier cells in a clinical trial of cellular immunological gene therapy. However, this procedure requires a long time to get a stable cell line and they are difficult to manipulate. Additionally, individual differences occur with the introduction of the gene and it is difficult to get a stable effect.

Disclosure

Problems to be Solved

The purpose of the present disclosure is to solve the above problems and to find new carrier cells that exhibit potent antitumor effects with cancer gene therapy using an oncolytic virus. Additionally, to establish a new cancer gene therapeutic method exhibiting a very potent antitumor effect using the carrier cells etc., and to provide a new therapeutic method using a cancer gene therapeutic drug.

Means for solving the problems

The applicants of the present disclosure have investigated solutions to the above problems and found that:

(1) a more potent antitumor effect can be obtained by using a specific cell line as the carrier cell in comparison to that of a conventional carrier cell, and

(2) inducing and raising a cytotoxic activity reaction through cytotoxic T lymphocytes (CTL reaction) within a living body by administering a virus for immunological treatment prior to administering the carrier cell infected with an oncolytic virus, gives a very potent *in vivo* antitumor effect, and accomplished the present disclosure.

That is, a cancer gene therapeutic drug of the present disclosure (in other words, a drug kit for cancer therapy) is a combination of: a virus for immunological treatment to be administered for inducing a CTL reaction within a living body and for administering to the carrier cell; and a carrier cell to be infected with an oncolytic virus before administering to the living body so as to make the oncolytic virus act on a tumor cell within the living body.

The virus for immunological treatment and the oncolytic virus of the present disclosure are preferably selected from viruses, such as, adenovirus, herpes virus, lentivirus, HIV virus, retrovirus, reovirus, vesicular stomatitis virus (VSV), and any other oncolytic virus. Among them, adenovirus gives a favorable result as shown later and could be preferred for use.

Preferably, the oncolytic virus of the present disclosure has a tumor specific promoter, according to the kind of cancers to be treated, including such as 1A1.3B promoter (IAI.3B promoter), midkine (MK) promoter, β-HCG promoter, SCCA1 promoter, cox-2 promoter, PSA promoter, or any other tumor specific promoter. Any oncolytic virus, capable of infection and proliferation in the target tumor cells, such as, adenovirus including its wild type, can be used in the present disclosure. Oncolytic viruses without a tumor specific promoter, such as an E1B gene deficient oncolytic adenovirus of ONYX Pharmaceuticals Inc. and an E1A gene partially deficient type Ad5-Δ24 adenovirus of University of Alabama at Birmingham (UAB), may be used.

The viruses for the immunological treatment used in the present disclosure are preferably a non-proliferative type and/or an inactivated type by UV irradiation, etc. Inactivation by UV irradiation, etc., may shorten the period between administering the virus for immunological treatment and administering the carrier cell.

The carrier cell of the present disclosure is preferably selected from A549 cells, 293 cells, SW626 cells, HT-3 cells (HT-III cells) and any other human derived cancer cell or normal cell. Further, other commercially available cell lines, such as, PER.C6 cells of Crucell may be used. The above mentioned A549 cells, 293 cells, SW626 cells and HT-3 cells gave favorable results as described later and are more preferable as the carrier cell. A549 cells are particularly favorable among them.

The cancer gene therapeutic drug of the present disclosure (a drug kit for cancer therapy) is a combination of the virus for immunological treatment and the carrier cell, or further including the oncolytic virus to produce a kit composed of three members. Additionally, the kits may include one or more substances of (1) - (4) shown below.

(1) Atelocollagen

(2) GM-CSF (granulocyte-macrophage colony stimulating factor) expression vector to be infected to the carrier cell before administration

(3) Iron preparations

(4) Porphyrin compounds (e.g. 5-aminolevulinic acid: ALA)

Together with administering the virus for immunological treatment, or before or after it, administering an irradiated tumor cell (a patient derived or a generally available one with similar antigen) is preferred for tumor vaccination (tumor immunization). The cancer gene therapeutic drug of the present disclosure may include such irradiated tumor cell for the tumor vaccination.

The cancer gene therapeutic method of the present disclosure comprises a step for administering a virus for immunological treatment to induce a CTL reaction within a living body to administer a carrier cell; and after a predetermined period, at least a single step of administering a carrier cell to be infected with an oncolytic virus before administering the cell to the living body so as to make the oncolytic virus act on a tumor cell within the living body.

The period from administering the virus for immunological treatment to administering the carrier cell in the cancer gene therapeutic method of the present disclosure is preferably set to be two weeks or more, and not more than 13 weeks (more preferably three weeks to four weeks). Preferably, (1) the dose of the virus for immunological treatment may be set between about 10^5 viral particles and 10^{11} viral particles for a patient with antibodies negative to the virus, while between about 10^2 viral particles and 10^7 viral particles for a patient with antibodies positive to the virus, (2) the dose of the oncolytic virus by using the carrier cell may be set between about

10^9 viral particles and 10^{14} viral particles, (3) the infection concentration of the oncolytic virus to the carrier cell may be set between about 0.1 viral particles/cell (hereinafter referred as "vp/cell") and 2,000 vp/cell. More preferably between five vp/cell and 500 vp/cell.

In the cancer gene therapeutic method of the present disclosure, adoption of one or more steps of the following (1) to (5) is preferred:

(1) Administering the carrier cell by intratumor injection,

(2) Administering atelocollagen together with the carrier cell,

(3) Administering the carrier cell infected with the oncolytic virus and a GM-CSF expression vector,

(4) Administering an iron preparation and/or a porphyrin compound [e.g. 5-aminolevulinic acid (ALA)] together with the carrier cell, and

(5) Administering a tumor cell for tumor vaccination together with, or before, or after, the virus for immunological treatment.

Effect of the Disclosure

The cancer gene therapeutic drug of the present disclosure is a combination of the two drugs composed of the virus for immunological treatment to be administered in advance and the carrier cell to be administered afterwards. Immunological treatment in advance by administering the virus, such as, adenovirus and then administering the carrier cell infected with the oncolytic virus provides a direct antitumor effect by infection of the oncolytic virus to the target tumor cells, and further induces the CTL reaction within the living body to the infected target cells, which provides a very potent *in vivo* antitumor effect.

Still further, use of a cell line, such as, A549 cells, which have high antitumor effects both *in vitro* and *in vivo*, provides a more potent antitumor effect in comparison to those of conventional carrier cells.

Brief description of drawings

Figure 1 shows the results of the inhibitory effect on the proliferation of ovarian cancer cells HEY using various cell lines as carrier cells, expressed by cell numbers at IC_{50}.

Figure 2 shows the inhibitory effect on the proliferation of oncolytic viruses without and together with carrier cells to the ovarian cancer cells HEY in the presence of antiviral antibodies, expressed by antibody titer of anti-adenovirus antibodies at IC_{50}.

Figure 3 shows the inhibitory effect on the proliferation of oncolytic adenovirus infected carrier cells (such as 293 cells) to the ovarian cancer cells HEY in the presence of antiviral antibodies, expressed by antibody titer of anti-adenovirus antibodies at IC_{50}.

Figure 4 shows the inhibitory effect on the proliferation to the ovarian cancer cells HEY using carrier cells of 293 cells, A549 cells, SW626 cells and HT-3 cells in the presence of antiviral antibodies, expressed by cell numbers.

Figure 5 shows the *in vivo* antitumor effect of oncolytic adenovirus infected carrier cells, using a tumor model with 10 - 15 mm massive tumor formed by subcutaneous transplantation of human ovarian cancer cells RMG-1 in a nude mouse.

Figure 6 shows the *in vivo* antitumor effect of oncolytic adenovirus infected carrier cells, using a tumor model with 10 - 15 mm massive tumor formed by subcutaneous transplantation of human ovarian cancer cells PA-1 in a nude mouse.

Figure 7 shows the *in vivo* antitumor effect of a cancer gene therapeutic drug of the present disclosure using a subcutaneous tumor model mouse [(C57BL/6×C3/Hel) F1 mouse] with normal immune system.

Figure 8 shows a photomicrograph of the cell fusion of A549 cells due to administration of adenovirus.

Figure 9 shows a control photomicrograph of A549 cells without administration of an adenovirus.

Figure 10 (a) shows the midkine (MK) mRNA expression by RT-PCR in human surgical samples of 1 - 21; Figure 10 (b) shows the midkine (MK) mRNA expression by RT-PCR of four cell lines of malignant gliomas in a similar manner; and Figure 10 (c) shows the midkine (MK) expression in each of the above cell lines by Western blot analysis.

Figure 11 shows the results of a comparative investigation of the promoter activity in each of the above cell lines using two different length midkine promoters.

Figure 12 (a) shows a schematic structure of the oncolytic adenovirus, having the midkine promoter, designed in the present disclosure; and Figure 12 (b) shows the E1A protein expression in each of the above cell lines infected with three kinds of adenoviruses by Western blot analysis.

Figure 13 (a) shows the results of a comparative investigation of the inhibitory effect of cancer cell proliferation with three kinds of adenoviruses; Figure 13 (b) shows the adenovirus E3 domain's influence on the inhibitory effect of proliferation; and Figure 13 (c) shows the antitumor effect of an adenovirus in a nude mouse subcutaneous transplantation model with a tumor of 5 mm diameter.

Figure 14 shows the results of the antitumor effect of carrier cells infected with an oncolytic virus having the midkine promoter on a massive tumor with a 10 - 15 mm diameter, compared with administering the oncolytic virus without carrier cells.

Figure15 shows the influence of Fe on the inhibitory effect of adenovirus AdE3-1A1.3B on the proliferation of ovarian cancer cells PA-1.

Figure 16 shows the influence of ALA on the inhibitory effect of adenovirus AdE3-1A1.3B on the proliferation of ovarian cancer cells PA-1.

Figure 17 shows the influence of the coexistence of Fe and ALA on the inhibitory effect of adenovirus AdE3-1A1.3B on the proliferation of ovarian cancer cells PA-1.

Figure 18 (a) shows the *in vivo* antitumor effect of a cancer gene therapeutic drug of the present disclosure (in the case of no UV irradiation treatment on the virus for immunological treatment) using a subcutaneous tumor model mouse [(C57BL/6×C3/He) F1 mouse] with normal immune system; and Figure 18 (b) shows the long term survival rate of each mouse used for the experiment.

Figure 19 show the interval between the administration of the virus for immunological treatment and the administration of the carrier cell. Figure 19 (a) shows the tumor volume in each mouse; and Figure 19 (b) shows the survival rate in each mouse group.

Figure 20 (survival curve) shows whether the administration interval in Figure 19 can be shortened by using adenovirus UV-Ad-β-gal inactivated by UV irradiation as the virus for immunological treatment.

Figure 21 (tumor growth curve) shows the administration rate of UV-Ad-β-gal used as the virus for immunological treatment.

Figure 22 show the tumor vaccination effect. Figure 22 (a) shows the tumor volume in each mouse and Figure 22 (b) shows the survival rate in each mouse group. The number (n) of each mouse group was five animals. In the Figure, control "SCC7" and "OVHM" show the subcutaneous transplantation of squamous epithelium cancer cells SCC7 or ovarian cancer cells OVHM at a concentration of 1×10^6 cells, followed by administering AdE3-1A1.3B infected carrier cells A549 to mice. "OVHM-RT + Ad-β-gal→SCC7, OVHM" shows the subcutaneous transplantation of SCC7 or OVHM, followed by administering AdE3-1A1.3B infected carrier cells A549 to mice, after the mice had tumor vaccination with irradiated OVHM and administering Ad-β-gal for induction of the CTL to the adenovirus.

Figure 23 (survival curve) shows the tumor vaccination effect with non-small-cell lung cancer A549 cells. The number (n) of each mouse group was 10 animals. In the Figure, control, "OVHM", shows subcutaneous transplantation of ovarian cancer cells OVHM at a concentration of 1×10^6 cells, followed by administering AdE3-1A1.3B infected carrier cells A549 to mice without tumor vaccination. "AdE3-1A1.3B-infected A549→OVHM" shows subcutaneous transplantation of ovarian cancer cells OVHM at a concentration of 1×10^6 cells, followed by administering AdE3-1A1.3B infected carrier cells A549 to mice, after the mice were subcutaneously vaccinated with 1×10^6 irradiated A549 cells infected with AdE3-1A1.3B.

Figure 24 shows the death rate caused by side effects with administering atelocollagen together with the carrier cell. In the Figure, "N" in parentheses is the number of mice.

Figure 25 shows the antitumor effect in the presence of anti-adenovirus antibodies with 1 to 3 administrations of adenovirus Ad-β-gal without UV inactivation treatment. Figure 25 (a) shows the tumor volume of each mouse and Figure 25 (b) shows the survival rate of each mouse group. A mixture of A549 cells and 293 cells was used as the carrier cells. The number (n) of each mouse group was five animals.

Figure 26 shows the antitumor effect in the presence of anti-adenovirus antibodies with 1 to 3 administrations of adenovirus Ad-β-gal without UV inactivation treatment. Figure 26 graph (a) shows the tumor volume of each mouse and Figure 26 (b) shows the survival rate of each mouse group. A549 cells were used as the carrier cells. The number (n) of each mouse group was five animals.

Figure 27 shows the *in vivo* antitumor effect of administering the carrier cells (A549 cells) infected with adenovirus AdE3-1A1.3B and a GM-CSF expression vector, and

administering atelocollagen together with the carrier cell. Figure 27 (a) shows the tumor volume of each mouse and Figure 27 (b) shows the survival rate of each mouse group. In the Figure, "×1", "×2" and "×3" in front of "Ad-β-gal" show 1, 2 and 3 administrations of adenovirus Ad-β-gal, respectively. The number (n) of each mouse group was five animals.

Figure 28 shows the *in vivo* antitumor effect of intraperitoneal administration of an iron preparation together with the carrier cell. Figure 28 (a) shows the tumor volume of each mouse and Figure 28 (b) shows the survival rate of each mouse group. In the Figure, "×1", "×2" and "×3" in front of "Ad-β-gal" show 1, 2 and 3 administrations of adenovirus Ad-β-gal, respectively. The number (n) of each mouse group was five animals.

Figure 29 shows the radiation dose in radiation exposure to the carrier cell A549 using a nude mouse.

Figure 30 shows the antitumor effect of carrier cells A549 irradiated with different doses, using (C57BL/6×C3/He) F1 mice with subcutaneous transplantation of OVHM.

Figure 31 shows the infection concentration (amount) of the oncolytic virus to the carrier cell A549.

Figure 32 shows the tumor vaccination effect with the ovarian cancer cells OVHM. Figure 32 (a) shows the tumor volume of each mouse and Figure 32 (b) shows survival rate of each mouse group. In the Figure, "A549" shows mice with three administrations of AdE3-1A1.3B infected carrier cell A549 without tumor vaccination and "OVHM-RT→A549" shows mice with three administrations of AdE3-1A1.3B infected carrier cells A549 after tumor vaccination with irradiated OVHM. The number (n) of each mouse group was five animals.

Best mode for carrying out the present disclosure

One embodiment of carrying out the present disclosure is given.

[1] Carrier cells and others used for a cancer gene therapeutic drug of the present disclosure.

The carrier cells used for a cancer gene therapeutic drug of the present disclosure are described below. The carrier cells can be selected, for example, from the following cells:

(1) A549 cells

(2) 293 cells

(3) SW626 cells

(4) HT-3 cells (HT-III cells).

Figure 1 shows the results of screening the carrier cells to find effective carrier cells to use for the cancer gene therapeutic drug. More specifically, the cancer gene therapeutic drug was prepared by infection of the oncolytic virus to candidate cell lines and the inhibitory effects on the cancer cell proliferation are shown. Adenovirus AdE3-1A1.3B (IAI.3B) was used as the oncolytic virus. The adenovirus AdE3-1A1.3B has an E1A gene and an E3 gene, and an ovarian cancer specific 1A1.3B promoter (IAI.3B promoter) as a tumor specific promoter upstream of the E1A gene. The adenovirus AdE3-1A1.3B was infected to various candidate cell lines at a concentration of 500 vp/cell for two days and then added to the ovarian cancer cells HEY on culture day two, and the inhibitory effect on the proliferation of the cancer cells HEY was investigated on culture day five.

The vertical axis of Figure 1 shows the cell number capable of obtaining 50% inhibitory effect (IC_{50}) on proliferation by the various candidate cell lines. The lower the number of cells indicates a higher inhibitory effect on proliferation. As shown in the Figure, the presently investigated cancer cell lines showed a high inhibitory effect on proliferation in the order of 293 cells, A549 cells, SW626 cells and HT-3 cells (HT- III cells). The 293 cells, A549 cells and SW626 cells exhibited about a 100-fold higher inhibitory effect on proliferation in comparison to PA-1 cells, which previously have been used as carrier cells. HT-3 cells also showed a similar high inhibitory effect on proliferation as that of SW626 cells.

In addition, an oncolytic adenovirus was infected in the above mentioned 293 cells, A549 cells, SW626 cells and HT-3 cells to prepare the cancer gene therapeutic drugs. Their inhibitory effect on cancer cell proliferation was investigated in the presence of a sufficient amount of anti-adenovirus neutralizing antibodies [Ab(+)]. As shown in Figure 4, all cancer gene therapeutic drugs which used the above mentioned four cell lines as carrier cells showed a potent inhibitory effect cancer cell proliferation. The conventional cancer gene therapeutic drug with a virus was difficult to use with frequent administrations because of the production of antibodies. However, the use of the above mentioned four cell lines as carrier cells provided a potent *in vitro* inhibitory effect on proliferation, despite of the presence of antibodies. In addition, A549 cells used as the carrier cell showed the most potent inhibitory effect on proliferation among the above mentioned four cell lines as shown in Figure 4. That is,

administering the adenovirus infected A549 cells in the presence of a sufficient amount of the anti-adenovirus neutralizing antibodies almost completely inhibited the proliferation of the target cancer cells, even with the presence of antibodies.

Additionally, *in vivo* experiments using a massive subcutaneous tumor (10 - 15 mm diameter) nude mouse model showed potent antitumor effect when the above mentioned A549 cells, 293 cells and SW626 cells were used as the carrier cell (see Figure 5 and Figure 6). The details of these experiments will be explained in the examples described later.

As shown above, the cancer gene therapeutic drug obtained by infection of the oncolytic virus to the carrier cell is capable of exhibiting in a high antitumor effect by the use of any one of the carrier cells, A549 cells, 293 cells, SW626 cells and HT-3 cells.

The above mentioned four cell lines are explained. A549 cells are derived from a non-small-cell lung cancer cell line, and their details are described, for example, in the article of Giard, D.J., Aaronson, S.A., Todaro, G.J., Arnstein, P., Kersey, J.H., Dosik, H., and Parks, W.P., *In vitro* cultivation of human tumors: establishment of cell lines derived from a series of solid tumors, J. Natl. Cancer Inst., **51**: 1417 - 1423, 1923. The 293 cells are derived from human embryonic kidney cells and have been used in many experiments and studies as adenovirus producing cells. The 293 cells are explained, for example, in the article of Xie QW, *et al.*, Complementation analysis of mutants of nitric oxide synthase reveals that the active site requires two hemes, Proc. Natl. Acad. Sci., USA, **93**: 4891 - 4896, 1996. The SW626 cells are a metastatic strain of colon cancer in the ovary and their details are described, for example, in the article of Fogh J., *et al.*, Absence of HeLa cell contamination in 169 cell lines derived from human tumors, J. Natl. Cancer Inst., **58**: 209 - 214, 1977. The HT-3 cells are uterine cervix squamous ep. cancer cells and their details are described, for example, in the article of Fogh J., *et al.*, Absence of HeLa cell contamination in 169 cell lines derived from human tumors, J. Natl. Cancer Inst., **58**: 209 - 214, 1977. These four cell lines are available from cell preserving organizations, such as, ATCC (American Type Culture Collection) and other commercially available cell depositories.

A549 cells have many advantages when used as the carrier cell, such as, (1) production of a high titer of oncolytic adenovirus and so tough that they can be easily handled, (2) most potent inhibition of proliferation of cancer cells in the presence of anti-adenovirus antibodies, (3) release of secretory granules due to infection of virus,, such as, adenovirus, because A549 cells

are derived from alveolar epithelial cell type II, and the property is favorable in the cancer gene therapy, and (4) resistance to cell elimination effect by CTL even after infection with adenovirus. Therefore, adoption of A549 cells is particularly preferable among the above mentioned four cell lines.

Multiple kinds of cells may be used as the carrier cells. A combination of A549 cells and 293 cells in the example described later revealed a potent cancer therapeutic effect. Concurrent use of multiple kinds of cells may utilize their respective characteristic features and advantages, and is preferable. For example, SW626 cells require a comparatively long period for adhesion and widely disperse into the surrounding areas with intraperitoneal administration, without restriction in the administered site, and are considered preferable for intraperitoneal therapy, such as, ovarian cancer. SW626 cells show the characteristic features of a late peak in their virus productivity than those of A549 cells and 293 cells, resulting in a comparatively longer period of function.

As described above, adoption of the above mentioned four cell lines (that is, A549 cells, 293 cells, SW626 cells and HT-3 cells) as the carrier cell is preferable. However, cells useable as the carrier cell are not limited to the above mentioned four lines and other cells, such as, PA-1 cells (e.g. particularly herpes virus used as an oncolytic virus), fibroblasts, and other human derived cancer cells, normal cells and patient derived cancer cells may be used as the carrier cell.

In the cancer gene therapeutic drug of the present disclosure, a conventional virus vector used for gene introduction may be used as an oncolytic virus to infect the carrier cell. Adenovirus, adeno-accompanying virus, herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), *Lentivirus*, such as, HIV virus (AIDS virus), retroviruses, such as, mouse leukemia virus, reovirus and vesicular stomatitis virus (VSV) can be exemplified and furthermore other oncolytic viruses may be used. The oncolytic virus is a proliferative virus vector and any virus that modifies the viral gene so as to specifically proliferate in the target tumor cells or tumor tissues, and fuse or kill target cells with cell lysis (cytolysis) action may be used. For example, an adenovirus having an E1A or E1B domain necessary for proliferation may be used.

The cancer gene therapeutic drug of the present disclosure can be applied to almost all malignant tumors and may include, for example, ovarian cancer, squamous epithelium cancers (e.g. uterine cervix carcinoma, cutaneous carcinoma, head and neck cancer, esophageal cancer

and lung cancer), digestive tract cancers (e.g. colon cancer, pancreatic cancer, hepatic cancer and gastric cancer), neuroblastoma, cerebral tumor, mammary cancer, testicular cancer and prostatic cancer. In addition, adoption of adenoviruses types 34 and 35, which are capable of infection to blood cells, gives the cancer gene therapeutic drug of the present disclosure applicable to blood malignant tumors.

Types of the tumor specific promoter to be introduced into the oncolytic virus may be selected according to the kind of target cancer. For example, 1A1.3B promoter for ovarian cancer, midkine promoter for, such as, cerebral tumor and malignant glioma, β-HCG promoter for testicular cancer, SCCA1 promoter and SCCA2 promoter for squamous epithelium cancers, CEA promoter for colonic cancer, PSA promoter for prostatic cancer and AFP promoter for hepatic cancer may be used. Naturally, other known tumor specific promoters, such as, the cox-2 promoter, which has a wide action spectrum and exhibits promoter activity to various malignant tumors, and other cancer specific promoters, such as, osteocarcine promoter may be used. The above mentioned midkine promoter may be used on various malignant tumors in addition to cerebral tumor and malignant glioma and has a wide action spectrum, as well as, the cox-2 promoter.

No specific limit is given for the length of each promoter sequence as far as it exhibits the tumor specific promoter activity. The above mentioned 1A1.3B promoter can be designed and prepared according to the disclosures in the pamphlet of International Publication No. 03/025190 and the literature, Cancer Research **63**, 2506 - 2512, 2003 and can be inserted in a virus genome. The above mentioned midkine promoter, β-HCG promoter and SCCA1 promoter can be designed and prepared according to the disclosures in the pamphlets of International Publication Nos. 02/10368, 01/90344 and 00/60068, respectively.

The above mentioned SCCA1 promoter is explained in detail in the article by Katsuyuki Hamada, Hiroto Shinomiya, Yoshihiro Asano, Toshimasa Kihana, Mari Iwamoto, Yasushi Hanakawa, Koji Hashimoto, Susumu Hirose, Satoru Kyo and Masaharu Ito, Molecular cloning of human squamous cell carcinoma antigen 1 gene and characterization of its promoter, Biochimica et Biophysica Acta, 91522 (2001) 1 - 8.

For example, preparation of an oncolytic adenovirus can be accomplished by insertion of a tumor specific promoter upstream of a primary gene E1A or E1B essential for the proliferation of adenovirus, or replacement with a primary gene E1A or E1B promoter. Similar insertion of

the tumor specific promoter upstream of a gene essential for the proliferation of virus is performed when viruses other than adenovirus, such as, HSV-1, HSV-2, retrovirus, reovirus and vesicular stomatitis virus (VSV) are used for the construction.

However, it is not necessary for the oncolytic virus to have the tumor specific promoter as far as it has specific proliferative property in the target tumor cells or tumor tissues. For example, oncolytic adenoviruses, such as, an E1B gene deficient type oncolytic adenovirus of ONYX Pharmaceuticals Inc. or an E1A gene partially deficient type Ad5-Δ24 adenovirus of University of Alabama at Birmingham (UAB) may be used. Thus, an oncolytic virus deficient of a tumor specific promoter also may be used. Further, a wild type adenovirus or a partially gene deficient type thereof may be used as the oncolytic virus.

Infection of the oncolytic virus to the carrier cell can be performed by conventional methods without restriction, for example, seeding of carrier cells on a plate, addition of the oncolytic virus at an amount sufficient to infect all cells, cultivation in RPMI medium and fetal calf serum (FCS) (-), under 95% O_2 and 5% CO_2 atmosphere at 37℃ for about 6 - 36 hours, which is simple and easy to operate. In the examples shown later, A549 cells, SW626 cells and HT-3 cells were cultured by this method and infected with the oncolytic virus, whereas 293 cells were cultured in DMEM medium and 10% FCS(+) and infected with the oncolytic virus. Fetal calf serum (FCS) is preferably kept under FCS(-) for 3 - 6 hours infection. Infection for an additional period is preferably carried out under FCS(-) for 3 - 6 hours and then FCS is added at a concentration of 10%.

The amount and period of oncolytic virus infection to the carrier cell may be suitably selected according to factors, such as, the volume and type of tumor to be treated, type and concentration of the carrier cell, type of oncolytic virus and administration method of the cancer gene therapeutic drug of the present disclosure. Examples are, without particular restriction,

for about 6 - 24 hours at about 5 - 250 vp/cell by intraperitoneal administration and for about 12 - 24 hours at about 5 - 500 vp/cell by intratumoral administration with the use of A549 cells;

for about 6 - 24 hours at about 250 - 2,000 vp/cell by intraperitoneal administration and for about 12 - 24 hours at about 100 - 500 vp/cell by intratumoral administration with the use of SW626 cells; and

for about 12 - 24 hours at about 5 - 50 vp/cell by intratumoral administration and for about 6 - 24 hours at about 0.1 - 10 vp/cell by intraperitoneal administration with the use of 293 cells.

As shown above, the amount and period of infection vary according to the types and administration methods of the carrier cells. The above examples set them within about 6 - 24 hours at about 0.1 - 2,000 vp/cell by intraperitoneal administration and about 12 - 24 hours at about 5 - 500 vp/cell by intratumoral administration.

The carrier cell may be stored without infection of the oncolytic virus, so they are available for the preparation as virus infected carrier cells after infection of the oncolytic virus. Storage of virus infected carrier cells is also possible by freezing the irradiated carrier cells infected with an oncolytic virus, and thawing them at the place of medical treatment. The storage of the carrier cells may be, for example, in liquid nitrogen or at about -150℃. On the other hand, the oncolytic virus may be kept, for example, at about -80℃.

Before use, the oncolytic virus is infected to the carrier cell by the aforementioned method and the resultant virus infected carrier cells can be administered "as is" or together with a conventional pharmaceutical carrier to a human body (or experimental animals, such as, mouse and rat). As shown later, simultaneous administering one or more combinations of atelocollagaen, an iron preparation and a porphyrin compound together with the carrier cell is preferable. Administering carrier cells infected with an oncolytic virus and a GM-CSF expression vector (virus vector double infected carrier cells) is also preferable.

The carrier cell is administered at a predetermined period after administering a virus for immunological treatment. When cancer cells are used as the carrier cell, radiation exposure before or after virus infection is preferable. Radiation exposure at a dose of 120 - 400 Gy, 20 - 40 Gy or 20 - 40 Gy was performed before the administering A459 cells, SW626 cells or HT-3 cells, respectively, as the carrier cell in the examples shown later. The dose of radiation exposure to A549 cells was investigated and no cell proliferation was observed at a dose of 120 Gy or over (Figure 29) and radiation exposure dose is preferably set between 120 Gy and 600 Gy (more preferably, between 150 Gy and 400 Gy).

The carrier cell may be preferably administered as a parenteral preparation; however, administration as an oral preparation may be also applicable. Administration as a parenteral preparation may be performed by an *in vivo* or *ex vivo* method. The dosage of *in vivo*

administration (in other words, the dosage of virus infected carrier cells) may be adjusted according to the volume and type of tumor, severity of disease, and patient's age and body weight, etc. For example, administration may be performed by intracavitary injections, such as, intravenous injection, intravenous drip infusion, intratumoral injection and intraperitoneal injection. Among them, the carrier cell is preferably administered by intratumoral injection. These injection preparations may be prepared by conventional procedures and general diluents, such as, a saline solution and a cell culture solution may be used. Furthermore, a bactericide, an antiseptic, a stabilizer, a tonicity agent and an analgesic may be added if necessary. No particular limit is given for the blending quantity of the virus infected carrier cells in these preparations and can be set to suitable levels.

The above mentioned virus infected carrier cells, of course, may be administered in several divided doses to patients or in several divided courses with optional sets of administration times and intervals.

As shown above, the dosage of virus infected carrier cells can be determined according to the volume and type of tumor, severity of disease, and patient's age and body weight, etc. Generally, the dosage of carrier cells can be set between about 10^7 cells and 10^{10} cells for one administration, whereas the dosage of oncolytic viruses through the carrier cell can be set between about 10^9 viral particles and 10^{14} viral particles for one administration.

The type of carrier cell may be suitably selected according to the type of cancer to be treated. The carrier cell may be modified by a gene recombinant technology, for example, an artificial expression of a specific protein on the surface of the carrier cell to increase the binding with the target tumor cells, or treatment, such as, infection of Sendai virus to the carrier cell.

The oncolytic virus can infect from the carrier cells to the target cells by cell to cell interaction, specifically proliferates in the tumor cells and exerts cell lysis (cytolysis) action of fusion or killing of the tumor cells. The cancer gene therapy with a virus was difficult to use with frequent administrations because of the production of antibodies, however, the carrier cells directly infect the target tumor cells by cell to cell interaction, and make frequent administrations possible and a potent antitumor effect can be expected.

[2] The cancer gene therapeutic drug of the present disclosure and its preferred application example

The cancer gene therapeutic drug of the present disclosure is a combination of: a virus for immunological treatment to be administered for inducing a CTL reaction within a living body to the administration of the carrier cell; and a carrier cell having been infected with an oncolytic virus before the administration so as to make the oncolytic virus act on a tumor cell within the living body. In other words, it is a combination of two drugs: a virus for immunological treatment administered in advance and then a carrier cell is administered. Administering the virus for immunological treatment, such as, adenovirus (immunization in advance) followed by administering the carrier cell infected with the oncolytic virus induces and raises the CTL reaction within the living body and can obtain a very potent *in vivo* antitumor effect.

The cancer gene therapeutic drug of the present disclosure showed a dramatic antitumor effect in a practical experiment using a syngenic model mouse with a normal immune system. Although the details will be described later, the ovarian cancer cells OVHM were subcutaneously transplanted to (C57BL/6×C3/He) F1 mice who were locally injected with carrier cells (A549 cells) infected with an oncolytic adenovirus having an ovarian cancer specific promoter. Mice immunized with adenovirus (Ad-β-gal) three months before the injection showed a marked antitumor effect 3 - 4 days after the start of administration, and the tumor had completely disappeared after nine days and lymph node metastasis had also diminished (see Figure 7 and Figure 18 and thereafter).

As mentioned above, more potent and dramatic antitumor effect was obtained in the experiment using the mice with normal immune system, producing antibodies. This result shows that the CTL reaction was induced and raised within the living body, by prior administering the virus for immunological treatment. The conventional cancer gene therapeutic drug with a virus was considered difficult to use with frequent administrations because of the production of antibodies, however, the cancer gene therapeutic drug of the present disclosure instead uses the immune system within the living body to attack the virus infected target tumor cells.

The virus for immunological treatment is preferably the same type as the oncolytic virus. Non-proliferative type and/or inactivated virus may be preferably used as the virus for immunological treatment. A non-proliferative type virus inactivated by treatment, such as, UV

irradiation to disrupt the DNA, may be more preferably used. For example, an adenovirus with an E1 domain deletion and/or inactivated by UV irradiation to disrupt DNA is favorably used as the virus for immunological treatment. A proliferative virus inactivated by UV irradiation, etc., may be used as the virus for immunological treatment.

In the examples described later, an adenovirus (Ad-β-gal) deficient of E1 domain, having LacZ gene encoding β-galactosidase (β-gal) under the control of cytomegalovirus (CMV) promoter, was used for the virus of immunological treatment. Of course, other viruses can be used as the virus for immunological treatment. For example, a proliferative adenovirus inactivated by UV irradiation may be used. A non-proliferative adenovirus having polyA sequence without integration of any gene (Ad-polyA), inactivated by UV irradiation, may be preferably used.

Dosages of the virus for immunological treatment in the cancer gene therapeutic drug of the present disclosure are suitably selected according to the patient's antibody titer to the virus, volume and type of tumor, severity of symptoms, and age and body weight of patient. Alteration of the dosage in accordance to whether the patient is positive or negative for the antibodies to the virus is preferred. For example, if adenovirus type 5 is used as the virus for immunological treatment and the oncolytic virus, the concentration of the virus for immunological treatment may be set between about 10^5 viral particles and 10^{11} viral particles for patients who are antibody negative (-), and may be set between about 10^2 viral particles and 10^7 viral particles for patients who are antibody positive (+). The method of administering the virus for immunological treatment is not restricted, although intracutaneous or subcutaneous injection is preferred.

In addition, the dose of each drug for animals, such as, mice and rats may be set at about 1/1,000 to that for a human body in consideration of the differences in body weight, for experimental administering the cancer gene therapeutic drug of the present disclosure.

The interval between administering the virus for immunological treatment and administering the carrier cell may be set between about two weeks and three months. The shorter period is more preferable. In the examples described later, inactivation of the virus for immunological treatment (adenovirus) by UV irradiation shortened the above mentioned interval to about three weeks or four weeks.

Dosages of virus infected carrier cells are as mentioned before and one dose of the oncolytic virus through the carrier cell may be set between about 10^9 viral particles and 10^{14} viral particles, and the infection concentration of the oncolytic virus to the carrier cell may be set between about 0.1 vp/cell and 2,000 vp/cell. Preferably between 5 vp/cell and 500 vp/cell. More preferably between 150 vp/cell and 400 vp/cell.

For tumor vaccination, administering tumor cells (cancer cells) together with, before or after, administering the virus for immunological treatment is preferable. That is, vaccination with the tumor cells (to enhance the immunological response in a living body to the target tumor cells by administering the tumor cells treated in advance with radiation exposure, ethanol or formaldehyde) is preferable together with, before or after immunization by the virus for immunological treatment.

A tumor cell derived from a patient is preferred for the tumor cells used for the above tumor vaccination (tumor immunization), and commonly available tumor cells with similar antigen may be used. The examples described later investigating the therapeutic effect to ovarian cancer (by OVHM) showed a favorable therapeutic effect by using a cancer cell (squamous epithelial cancer cells SCC7 and lung cancer cells A549), for tumor vaccination that is different from the target cancer cell to be treated.

In the above tumor vaccination (tumor immunization), no particular limited concentration of tumor cell is given. For example, it may be set between about 10^5 cells and 10^{10} cells. The radiation exposure dose to the tumor cells is preferably set between 120 Gy and 600 Gy. More preferably between 200 Gy and 500 Gy. The preferred administration method is intracutaneous injection or subcutaneous injection.

Furthermore, administering an iron preparation and/or a porphyrin compound may be used to enhance the viral productivity in the cancer to be treated. Porphyrin compounds, such as, 5-aminolevulinic acid (ALA), hematoporphyrin and photofirin are exemplified. As iron preparations, ferrous sulfate ($FeSO_4$) and ferrous citrate for oral administration, and chondroitin sulfate iron and sugar containing iron oxides for intravenous administration may be exemplified. The administration method is not limited, although an injection preparation or oral preparation is preferable, together with the cancer gene therapeutic drug of the present disclosure.

Practically, administering an iron (Fe) preparation and/or 5-aminolevulinic acid (ALA) could markedly enhance the inhibitory effect of the oncolytic adenovirus AdE3-1A1.3B on cancer cell proliferation (see Figures 15 - 17 and Figure 28).

Administering atelocollagen (product prepared by cleavage of only telopeptide bond of collagen by pepsin treatment, etc., making the molecular weight small and water soluble) together with the carrier cell is also preferred. As shown in examples described later, administering atelocollagen and the carrier cell simultaneously, dramatically reduced the death rate caused by side effects (Figure 24). This might be caused by inhibition of dispersion of the oncolytic adenovirus and a block against anti-adenovirus antibodies that was produced by the atelocollagen.

Therefore, administering atelocollagen and the carrier cell simultaneously can suppress side effects and realize a high dose administration of the oncolytic virus. Commercially available atelocollagen (e.g. a product of Koken Co., Ltd.) or a product prepared by treatment of collagen with pepsin may be used. Atelocollagen is preferably administered by mixing it with the injection solution together with the carrier cells. A concentration of 0.01 - 3.0% (w/v) in the solution is considered to exhibit a sufficient effect. (Examples described later showed a sufficient effect at a low concentration of 0.1 - 0.2% (w/v) in the solution).

Furthermore, as described earlier, administering the carrier cells doubly infected with an oncolytic virus vector and a GM-CSF expression virus vector is preferred to enhance the immune response. (Or, it is possible to simultaneously administer two kinds of carrier cells each infected with one of the above virus vectors.)

It is preferred that the GM-CSF expression vector is the same kind of virus vector as the oncolytic virus. For example, when adenovirus is used as the oncolytic virus, one may use, for the GM-CSF expression vector, an adenovirus deficient of E1 domain and a GM-CSF gene encoding granulocyte-macrophage colony stimulating factor (GM-CSF).

Using the GM-CSF expression vector, as well as, the oncolytic virus, a total concentration of both virus vectors to the carrier cell may be set between 5 vp/cell and 2,000 vp/cell.

Application of the GM-CSF expression vector showed a very excellent cancer therapeutic effect (Figure 27).

Instead of the GM-CSF expression vector, GM-CSF protein may be mixed in an injection solution together with the carrier cells, or the protein's administration by intravenous administration may be taken in consideration.

Methods for use of the cancer gene therapeutic drug of the present disclosure are, of course, not restricted to the methods described above and various methods for use are available. For example, the cancer gene therapeutic drug of the present disclosure may be concurrently used with other anticancer agents or a radiation therapy to enhance the infectivity of the oncolytic virus.

Examples of the preferred use of the cancer gene therapeutic drug of the present disclosure will be given by dividing them into (1) patients that are negative for the antibodies to the virus for immunological treatment and (2) patients that are positive for the antibodies to the virus for immunological treatment.

In the above case (1), non-proliferative adenovirus inactivated by UV irradiation, as described earlier, may be used for the virus for immunological treatment. The amount is about 10^5 vp to 10^{11} vp. Patient's derived tumor cells (cancer cells) irradiated at about 200 Gy for tumor vaccination may be administered at 10^5 cells to 10^{10} cells together with the virus for immunological treatment. The virus for immunological treatment and the tumor cell may be administered by intracutaneous or subcutaneous injection.

About 3 - 4 weeks after administering the virus for immunological treatment and the tumor cell, the carrier cell may be administered by intratumoral administration. The dose of the carrier cells may be set about 1×10^7 to 1×10^{10} cells for one administration. A549 cells, irradiated at about 150 Gy to 400 Gy and then administered, may be used as the carrier cell. The adenovirus may be used for the oncolytic virus and the GM-CSF expression vector, and may be infected to the carrier cell at about 250 vp/cell and 5 - 20 vp/cell, respectively. Atelocollagen may be mixed with an injection solution at a concentration of about 0.1 - 0.2% and then administered. Simultaneously, an iron (Fe) preparation may be intravenously administered at a dose about 40 - 100 mg. ALA may be simultaneously administered into the tumor at a dose of 2 - 2,000 mg.

As mentioned above, the carrier cells, etc., may be administered once. However, the carrier cells, etc., may be administered 1 - 6 times. Administering multiple times may be carried out in consecutive days or at intervals of 2 - 3 days.

In the above case (2), patients with positive antibodies, the cancer gene therapeutic drug of the present disclosure may be administered in a similar manner with that of the above case (1), except for setting the amount of the virus for immunological treatment at about 10^5 vp or less.

Practical examples of the cancer gene therapeutic drug of the present disclosure are, such as,

(1) a combination of the virus for immunological treatment and the carrier cell,

(2) a combination of the virus for immunological treatment, the carrier cell and the oncolytic virus for the infection to the carrier cell,

(3) a combination with atelocollagen added to the above combinations (1) or (2),

(4) a combination with GM-CSF expression vector added to the above combinations (1) - (3),

(5) a combination with an iron preparation and/or a porphyrin compound added to the above combinations (1) - (4) to enhance the viral productivity,

(6) a combination with the tumor cell for tumor vaccination added to the above combinations (1) - (5), and

(7) a combination with the necessary compounds for storage, infection and culture, and preparation of medical preparations (e.g. a reagent, a buffer and an enzyme), or vessels (e.g. for reaction, infection and culture, and storage) added to the above combinations (1) - (6).

Examples

Examples of the present disclosure are explained referring the drawings, however, the scope of the present disclosure is not restricted by these examples.

[Example 1: Screening of the carrier cell and antitumor effect in the presence of antibodies]

The following experiments were carried out to screen cancer cell lines which exhibit a potent inhibitory effect on cancer cell proliferation when they are used as the carrier cell.

Adenovirus AdE3-1A1.3B (IAI.3B) was used as the oncolytic virus for the infection to the carrier cell. The adenovirus AdE3-1A1.3B has an E1A gene and an E3 gene, and an ovarian cancer specific 1A1.3B promoter (IAI.3B promoter) as a tumor specific promoter upstream of the E1A gene. The adenovirus AdE3-1A1.3B was infected to various carrier cells at a

concentration of 500 vp/cell for two days, and then the carrier cells were added to an ovarian cancer cell line HEY on culture day two and their *in vitro* inhibitory effects on proliferation were investigated on culture day five.

The results of the above experiment are shown in Figure 1. The vertical axis of the graph shows cell numbers capable to obtain a 50% inhibitory effect (IC_{50}) on proliferation for each cell line, and a low cell number shows a higher inhibitory effect on proliferation. As shown in the Figure, the cancer cell lines investigated in the present experiment showed high antitumor effects in the order of 293 cells, A549 cells, SW626 cells and HT-3 (HT-III) cells. The 293 cells, A549 cells and SW626 cells exhibited about a 100-fold higher inhibitory effect on proliferation in comparison to PA-1 cells which previously have been used as the carrier cells. HT-3 cells also exhibited a similar high inhibitory effect on proliferation as that of SW626 cells.

Then, the difference in the inhibitory effect on proliferation, in the presence of antiviral antibodies, was examined between using only the oncolytic virus, and a combination of the oncolytic virus and the carrier cell. As the carrier cell, a 293 cell was used and the above mentioned adenovirus AdE3-1A1.3B was infected for two days. The resultant adenovirus AdE3-1A1.3B infected 293 cells and supernatant (AdE3-1A1.3B 293 cell+SUPT) were placed in a 12-well plate in the presence of the anti-adenovirus antibodies. In each well, about 50,000 cells of the ovarian cancer cell line HEY had been cultured from the preceding day. The anti-adenovirus antibodies were prepared by dilution of the antibodies with 600-fold antibody titer to various antibody titers. In the case of only the oncolytic virus, the adenovirus AdE3-1A1.3B was administered in the 12-well plate at a concentration of 1,000 vp/cell, in the presence of the anti-adenovirus antibodies. At culture day five, the respective inhibitory effect on the proliferation of cancer cells (HEY cells) were investigated.

The results of the above experiment are shown in Figure 2. The vertical axis of the graph shows the dilution rate of the anti-adenovirus antibodies at 50% inhibitory effect (IC_{50}) on proliferation. In other words, a 50% inhibitory effect on proliferation was obtained with 293 cells even at about 5-fold dilution rate (120-fold antibody titer), whereas a 50% inhibitory effect on proliferation was obtained with only the adenovirus at about 600-fold dilution rate (1-fold antibody titer). As shown above, the carrier cell exhibited an inhibitory effect on proliferation even under the condition of a high antibody titer.

Similarly, the inhibitory effect on proliferation of HEY cells was investigated in the presence of anti-adenovirus antibodies in the following conditions,

(1) adenovirus infected 293 cell and its supernatant (AdE3-1A13B 293 cell+SUPT),

(2) a cell supernatant containing adenovirus (AdE3-1A13B, SUPT),

(3) a filtered [0.2 μm] cell supernatant containing adenovirus (AdE3-1A13B, SUPT, filter), and

(4) only the adenovirus (AdE3-1A13B).

The results are shown in Figure 3. The vertical axis of the graph shows a dilution rate of the anti-adenovirus antibodies at a 50% proliferation inhibitory rate (IC_{50}). As shown in the Figure, a more potent antitumor effect was obtained in comparison to the other conditions when the carrier cell (293 cell) was used.

The inhibitory effect on the proliferation of the cancer cells HEY was investigated in the carrier cells 293 cells, A549 cells, SW626 cells and HT-3 cells; in either the presence [Ab(+)] or absence [Ab(-)] of the anti-adenovirus antibodies having a 600-fold antibody titer. The results are shown in Figure 4. The vertical axis of the graph shows the number of cancer cells on culture day five. As shown in the Figure, the most potent inhibitory effect on proliferation was obtained when A549 cells were used as the carrier cells in four types of cells. That is, administering adenovirus infected A549 cells in the presence of a sufficient amount of anti-adenovirus neutralizing antibodies almost completely inhibited the proliferation of the target cancer cells despite of the presence of the antibodies. The other three types of cells also showed sufficient inhibitory effect on proliferation in the presence of the antibodies.

The cancer gene therapy with a virus was considered to be difficult with multiple administrations because of the production of neutralizing antibodies to the virus. However, application of the carrier cell established direct infection to the target cancer cells by cell to cell interaction and multiple administrations became possible. Furthermore, application of the above mentioned four types of cells as the carrier cell provided potent antitumor effect.

[Example 2: *In vivo* antitumor effect in a nude mouse subcutaneous tumor model]

The *in vivo* antitumor effect of each carrier cell infected with the above mentioned adenovirus AdE3-1A1.3B was investigated using a nude mouse subcutaneous tumor model. In the experiment, human ovarian cancer cells RMG-1 were subcutaneously transplanted into

5-week-old nude mouse. After four weeks, each carrier cell was injected six times into a massive tumor of about 10 - 15 mm diameter and the change in the tumor volume was observed. The results are graphically shown in Figure 5. In the graph, the black squares show the "control" which are the results of injecting PBS buffer six times into the tumor; the black circles show "AdE3-1A1.3B" which are the results of administering 1×10^{10} viral particles of the adenovirus AdE3-1A1.3B per mouse; the black triangles show the results of administering 1×10^{7} SW626 cells infected with the adenovirus AdE3-1A1.3B at 250 vp/cell per mouse; the black diamonds show the results of administering 1×10^{7} 293 cells infected with the adenovirus AdE3-1A1.3B at 25 vp/cell per mouse; and the white squares show the results of administering 1×10^{7} A549 cells infected with the adenovirus AdE3-1A1.3B at 50 vp/cell per mouse. As shown in the Figure, when 293 cells and A549 cells were used as the carrier cells, they showed complete disappearance of the massive tumor (10 - 15 mm diameter) 50 days after the treatment. SW626 cells showed a 98% inhibitory effect on proliferation.

A similar experiment as shown above was carried out by subcutaneous transplantation of human ovarian cancer cells PA-1 into 5-week-old nude mouse. The results are shown in Figure 6. As shown in the Figure, the massive tumor (10 - 15 mm diameter) completely disappeared by using 293 cells and A549 cells as the carrier cells. SW626 cells showed complete disappearance of the tumor in four out of five mice.

[Example 3: *In vivo* antitumor effect in subcutaneous tumor model mouse with normal immune system]

The *in vivo* antitumor effect of the cancer gene therapeutic drug of the present disclosure was investigated using (C57BL/6×C3/He) F1 mice with a normal immune function. In the experiment, each antitumor effect was investigated with the following conditions.

(1) The ovarian cancer cells OVHM were subcutaneously transplanted to the syngenic model mouse. After 10 days or more, A549 cells were infected with the adenovirus AdE3-1.3B at a concentration of 250 vp/cell, followed by radiation exposure, were administered six times into a formed 5 - 10 mm tumor.

(2) A 7-week-old syngenic model mouse was immunized in advance with an adenovirus for immunological treatment. After three months, the ovarian cancer cells OVHM were subcutaneously transplanted in a similar manner with that of (1) and then, after 10 days or more,

A549 cells infected with the adenovirus AdE3-1A1.3B at a concentration of 250 vp/cell, followed by radiation exposure, were administered six times into a tumor.

(3) PBS buffer was administered six times into a tumor as a control.

The results of the above experiment are shown in Figure 7. In the graph, the black squares show the "control" which are the results of the above condition (3); the black circles show "AD(-)→A549" which are the results of the above condition (1), without administering the adenovirus for immunological treatment; and the black triangles show "Ad(+)→A549" which are the results of the above condition (2), with administering the adenovirus for immunological treatment.

A non-proliferative type adenovirus having no E1 gene was used for the adenovirus for immunological treatment. More specifically, it was an adenovirus with an inserted LacZ gene downstream of the CMV promoter. As shown by the Figure, the above condition (1), without prior immunization by adenovirus, showed 20% antitumor effect in comparison to the control, while the above condition (2), with prior immunization by adenovirus, showed a marked antitumor effect 3 - 4 days after the start of administration and the tumor was completely diminished after nine days with a disappearance of lymph node metastasis. As shown by the example, the potent and dramatic antitumor effect in mice with a normal immune system, despite their antibody production, might be caused by the induction and raising of the CTL reaction within the living body due to administering the adenovirus for immunological treatment.

The oncolytic adenovirus is infected from the carrier cells to the target tumor cells by cell to cell interaction, specifically it proliferates in the tumor cells and is considered to exert cell lysis (cytolysis) action to fuse and/or kill the target tumor cells. The cancer gene therapeutic drug of the present disclosure is considered to induce a potent CTL reaction within the living body by prior administering the adenovirus for immunological treatment, which eliminates the oncolytic adenovirus infected target tumor cells and induces complete natural elimination of the adenovirus infected tumor cells.

One manner of infection of the adenovirus to the target tumor cell is believed to be a cell fusion caused by the adenovirus. Figure 8 is a photomicrograph of cells after overnight culture, after being 10,000 vp/cell of the adenovirus inactivated by UV irradiation were placed into a well with A549 cells. As shown by the arrow marks in the Figure, administering the adenovirus

caused cell fusion and multinucleated cells were sporadically observed. No such cells were observed in A549 cells without administering the adenovirus (see Figure 9).

Predicted infection manners, other than cell fusion, are cell adhesion to the target cells by the carrier cells and infection of the adenovirus to the target tumor cells by a local burst with a carrier cell fragment including the adenovirus. In any way, proliferation of an adenovirus having a tumor specific promoter in the adenovirus infected target tumor cells may lead to presentation of a potent antigen (or, a cancer specific peptide recognized as an antigen secondarily), and the tumor cells may be eliminated by the CTL reaction.

[Example 4: Antitumor effect by the use of a midkine promoter]

The antitumor effect of using a midkine promoter was investigated. Figure 10 (a) shows the midkine (MK) mRNA expressions in human surgical samples 1 - 21 by RT-PCR. As shown by the Figure, excessive expression of the midkine mRNA was observed in malignant gliomas, such as, glioblastoma and anaplastic astrocytoma, and in diffuse astrocytoma. Thus, the excessive expression of midkine is observed in many cancers, such as, cerebral tumors.

Figure 10 (b) shows the midkine mRNA expression by RT-PCR in four cell lines of malignant gliomas in a similar manner as shown above. As shown by the Figure, no expression was observed in U87MG and excessive expression of the midkine mRNA was observed in U251MG, LN319 and U373MG.

Figure 10 (c) shows the midkine protein expression in the above mentioned each cell line by Western blot analysis. No expression was found in U87MG, as well as, mRNA. Excessive expression of the midkine protein was observed in U251MG, LN319 and U373MG.

Then, a promoter assay of the midkine was performed. In the experiment, activity of two different length midkine promoters (600 bases and 2,300 bases) was compared. Plasmids (pGL3-MK600 and pGL3-MK2300) with a luciferase gene inserted downstream of the respective promoters were introduced to each of the above mentioned cell lines and their respective luciferase activity was investigated to evaluate the promoter activity. The results shown in Figure 11 revealed a higher promoter activity in the 600 base sequence length than in the 2,300 base sequence length in the malignant glioma cell line.

Figure 12 (a) shows a schematic diagram of the oncolytic (cytolysis type) adenovirus structure having a midkine promoter designed in the present experiment. The midkine

promoter having a 600 base sequence or a 2,300 base sequence was introduced at the site of 552 bp.

Figure 12 (b) shows the E1A protein expression in the above mentioned each cell line infected with three types of adenoviruses by Western blot analysis. As shown in the Figure, expression of E1A protein of the adenovirus was observed in midkine expressing U251MG, LN319 and U373, by the infection of adenovirus (AdMK600) having a 600 base length midkine promoter. Expression of E1A protein was observed in all cells, including normal brain cells, by wild type adenovirus (AdWild) and no expression of the E1A protein was observed in all cells with the control virus AdLacZ.

Figure 13 (a) shows the results of a comparative investigation of the inhibitory effect on the proliferation of cancer cells by three types of adenoviruses. Wild type adenovirus (AdWild) showed a potent inhibitory effect on proliferation in all cells, whereas adenoviruses (AdMK600 and AdMK2300) having the midkine promoter showed the inhibitory effect on proliferation only in midkine expressing U251MG, LN319 and U373MG. These results were well correlated with the results of midkine mRNA expression and promoter activity. The adenovirus AdMK600 showed a more potent inhibitory effect on proliferation than AdMK2300 which has a 2,300 base sequence length.

Figure 13 (b) shows the adenovirus E3 domain's influence on the inhibitory effect on proliferation. As shown in the Figure, AdMK600 having an E3 domain exhibited about a 10-fold potent inhibitory effect on proliferation than an adenovirus having no E3 domain (AdMK600-ΔE3).

Figure 13 (c) shows the antitumor effect of an adenovirus in a nude mouse subcutaneous transplantation model with about a 5 - 100 mm diameter tumor. In the Figure, the black diamonds show the results of administering the wild type adenovirus AdWild; the black squares show the results of administering the adenovirus AdMK600 having a midkine promoter; the black triangles show the results of administering the adenovirus Ad-β-gal with an inserted LacZ gene; and the black circles show the results of administering only PBS buffer. As shown in the Figure, only the wild type adenovirus showed antitumor effect in the U87MG without midkine expression. In the U373MG expressing midkine, AdMK600, as well as, AdWild produced a complete disappearance of tumor. No marked difference was observed between the control of injections with only PBS buffer and injections with AdLacZ.

Furthermore, an adenovirus having the above mentioned midkine promoter (Ad-MK600) was infected into the carrier cells and the antitumor effect of the virus infected carrier cells was compared to administering only Ad-MK600. In the experiment, 293 cells and A549 cells were used as the carrier cells. The above mentioned U373MG cells were transplanted into 5-week-old nude mice to give a 10 - 15 mm massive tumor after three weeks. The virus infected carrier cells or only Ad-MK600 were administered and the tumor volume was compared after four weeks. The results are shown in Figure 14. In the Figure, "Ad-MK600" shows the results of administering only Ad-MK600; and "293" and "A549" show the results of administering the virus infected carrier cells using 293 cells and A549 cells as the carrier cells, respectively. As shown in the Figure, administering the virus infected carrier cells showed complete disappearance of the tumor. Administering only Ad-MK600 showed almost no difference with that of the control.

Practically, favorable therapeutic effects on the ovarian cancer and malignant glioma were observed by application of carrier cells, such as, A549 cells and 293 cells and an adenovirus having a 1A1.3B promoter or midkine promoter as the oncolytic virus. The midkine promoter can be used for various malignant tumors in addition to malignant glioma and is considered effective in the cancer therapy of other than malignant glioma.

[Example 5: Influences of Fe and ALA on the inhibitory effect on proliferation of adenovirus AdE3-1A1.3B]

Ovarian cancer cells HEY were cultured in a 12-well plate at a concentration of 10,000 cells/well and $FeSO_4$ was added at a concentration of 50 μg/ml, 5 μg/ml, 0.5 μg/ml or 0 μg/ml on the following day and the cytolysis type adenovirus AdE3-1A1.3B was added to all wells. The inhibitory effect on proliferation of the adenovirus was evaluated by IC_{50} after five days. The results are shown in Figure 15. In the Figure, the vertical axis shows the relative concentration (vp/cell) of viruses at IC_{50} in each condition. As shown in the Figure, administering 50 μg/ml of $FeSO_4$ and the adenovirus showed about 20-fold, and administering 5 μg/ml of $FeSO_4$ and the adenovirus showed about an 8-fold inhibitory effect on proliferation, respectively, to that of only adenovirus administration.

Next, the ovarian cancer cell line HEY was cultured in a 12-well plate at a concentration of 10,000 cells/well and 5-aminolevulinic acid (ALA) was added at a concentration of 50 μg/ml,

5 μg/ml, 0.5 μg/ml or 0 μg/ml on the following day and the cytolysis type adenovirus AdE3-1A1.3B was added to all wells. The inhibitory effect on proliferation of the adenovirus was evaluated by IC_{50} after five days. The results are shown in Figure 16. In the Figure, the vertical axis shows relative concentration (vp/cell) of viruses at IC_{50} in each condition. As shown in the Figure, administering 50 μg/ml of ALA and the adenovirus showed about a 100-fold inhibitory effect on proliferation to that of only adenovirus administration.

Furthermore, the ovarian cancer cell line HEY was cultured in a 12-well plate at a concentration of 10,000 cells/well and $FeSO_4$ was added at a concentration of 50μg/ml, 5μg/ml, 0.5μg/ml or 0μg/ml on the following day. Additionally, the cytolysis type adenovirus AdE3-1A1.3B and 50μg/ml of 5-aminolevulinic acid (ALA) were added to each well. Only the adenovirus was added to the control. The inhibitory effect on proliferation of the adenovirus was evaluated by IC_{50} after five days. The results are shown in Figure 17. In the Figure, the vertical axis shows the relative administration concentration (vp/cell) of viruses at IC_{50} in each condition. As shown in the Figure, concurrent administration of 50 μg/ml of $FeSO_4$, 50 μg/ml of ALA and the adenovirus showed about a 1,000-fold inhibitory effect on proliferation to that of only adenovirus administration. Concurrent administration of 5 μg/ml of $FeSO_4$, 50 μg/ml of ALA and the adenovirus showed about a 700-fold inhibitory effect on proliferation to that of only adenovirus administration, and concurrent administration of 0.5 μg/ml of $FeSO_4$, 50 μg/ml of ALA and the adenovirus showed about a 200-fold inhibitory effect on proliferation to that of only adenovirus administration.

As mentioned above, it was found that ALA and Fe markedly enhance the inhibitory effect on proliferation of the oncolytic adenovirus AdE3-1A1.3B. ALA and Fe elevate the infectivity of adenovirus and the amount of virus production, because the β-gal assay revealed increased infectivity of adenovirus and the PFU assay revealed an increased amount of adenovirus production. That is, ALA and Fe can enhance the antitumor effect, because they can increase the infectivity of the adenovirus to cancer cells and the amount of virus production within the cells.

ALA is known to be a porphyrin metabolite taken up into cancer cells and its metabolite protoporphyrin IX is likely to be accumulated by the porphyrin metabolism. This compound has photo-sensitizing effect and it can be utilized for the photodynamic therapy (PDT) of superficial cancer, together with an excimer dye laser.

The above mentioned protoporphyrin IX binds with Fe to give a heme and forms heme proteins, such as, cytochrome in cells. The heme proteins are involved in the respiratory system in intracellular mitochondria, ATP production and protein synthesis. Thus, the heme proteins are involved in protein synthesis, including production of the adenovirus if the adenovirus infected. Therefore, promotion of the porphyrin metabolism may lead to the increased adenovirus production.

The cancer gene therapeutic drug of the present disclosure, as well as the cancer gene therapy of the present disclosure, can further increase the therapeutic effect by concurrent use of Fe and/or porphyrin compounds, such as, ALA. That is, concurrent use of Fe and/or porphyrin compounds, such as, ALA enhances antitumor effect, even under an infection suppressive condition in the presence of antibodies by acceleration of the CTL response caused by the increased adenovirus production in the target cells. Concurrent use of Fe and/or porphyrin compounds can enhance the antitumor effect not only in a syngenic mouse model with immune system but also in a human body.

In the cancer gene therapy using the oncolytic virus, concurrent use of Fe and/or porphyrin compounds, such as, ALA is expected to enhance the therapeutic effect, even if the carrier cells are not used.

[Example 6: Investigation for optimization of cancer therapy using the cancer gene therapeutic drug of the present disclosure]

The following a series of experiments were carried out to optimize the cancer therapy using the cancer gene therapeutic drug of the present disclosure.

At first, an investigation of the *in vivo* antitumor effect of the cancer gene therapeutic drug of the present disclosure was performed in a similar manner to the experiment shown in Figure 7 using a subcutaneous tumor model mouse [(C57BL/6×C3/He) F1 mice] with a normal immune system. In the experiments, (C57BL/6×C3/He) F1 mice of 5-week-old were immunized in advance by administering the virus for immunological treatment, and twelve weeks later, ovarian cancer cells OVHM were subcutaneously transplanted at a concentration of 1×10^6 cells per mouse to form a 5 - 10 mm tumor. Then, A549 cells (Ad-A549) infected with the above mentioned adenovirus AdE3-1A1.3B at a concentration of 250 vp/cell were administered into the tumor. A non-proliferative adenovirus having no E1 gene was used as the

virus for immunological treatment, more specifically, adenovirus Ad-β-gal with an inserted LacZ gene downstream of the CMV promoter, without inactivation by UV irradiation, was used and intracutaneously administered at a concentration of 1×10^{10} vp per mouse. The carrier cells, A549 cells, were irradiated at a dose of 200 Gy and were administered into the tumor of the mouse at a concentration of 5×10^{6} cells per treatment, six times in total.

The results of the above experiment are shown in Figures 18 (a) and (b). In each graph, "Ad-β-gal→Ad-A549" shows the results of the above mentioned experiment, "Ad-A549" shows the results of administering only the carrier cells, "Ad-β-gal" shows the results of administering only the Ad-β-gal for treatment (not as the virus for immunological treatment), and "control" shows the results of administering PBS buffer. Number (n) of mice in each group was five animals. Figure 18 (a) shows the tumor volume in each mouse for a comparatively short period and Figure 18 (b) shows the survival rate of the mice in each group for a long period. As shown by these figures, a potent *in vivo* antitumor effect was observed in "Ad-β-gal→Ad-A549".

The interval between administering the adenovirus for immunological treatment and the carrier cell was investigated. This experiment was carried out similar to that in Figure 18, except for the various changes in the intervals of administration and infection of adenovirus AdE3-1A1.3B with carrier A549 cell at 50 vp/cell.

The results of the above mentioned experiments are shown in Figures 19 (a) and (b). In each figure, "2-4w", "5-9w", "10-15w" and "16-22w" show the results of experiments with above mentioned administration intervals of 2 - 4 weeks, 5 - 9 weeks, 10 - 15 weeks and 16 - 22 weeks, respectively. Number (n) of mice in each group was five animals. As shown by these Figures, the best antitumor effect was obtained when the above mentioned administration interval was set at 10 - 15 weeks. As shown by the present experiments, when the adenovirus Ad-β-gal, without inactivation, was administered as the virus for immunological treatment, the CTL reaction by T cells was considered to become predominant at about 10 - 15 weeks after the administration, compared with the suppression of infection due to neutralizing antibodies.

The above mentioned administration interval is preferred to be short in consideration of the clinical application. Then, it was investigated whether the above mentioned administration interval could be shortened by using inactivated adenovirus Ad-β-gal as the virus for immunological treatment. As shown in Figure 20, it was found that when the UV inactivated adenovirus UV-Ad-β-gal was used as the virus for immunological treatment, the administration

interval of four weeks or three weeks showed favorable antitumor effects, that is, inactivation of the virus for immunological treatment can be shortened to an administration interval of about 3 - 4 weeks.

The above mentioned experiments were carried out similar to those of experiments shown in Figure 18, except for the following points; inactivated adenovirus UV-Ad-β-gal was used as the virus for immunological treatment, the above UV-Ad-β-gal was intracutaneously administered at a concentration of 1×10^7 vp per mouse, the administration intervals were set to three weeks, four weeks, five weeks or six weeks, and the carrier A549 cell was infected with the adenovirus AdE3-1A1.3B at a concentration of 50 vp/cell. Number (n) of mice in each group was 10 animals.

Figure 21 shows the virus dosage when UV-Ad-β-gal was used as the virus for immunological treatment. In this experiment, the concentration of the UV-Ad-β-gal was changed to a range of 1×10^6 vp to 1×10^{11} vp. The experiment was carried out similar to Figure 20, except the administration interval was set at six weeks. The result showed that the concentration of UV-Ad-β-gal set at 1×10^7 vp gave the best antitumor effect. (From this result, the concentration of UV-Ad-β-gal was set at 1×10^7 vp in the experiment shown in Figure 20).

Figures 22 (a) and (b) show the effect of tumor immunization (tumor vaccination). The above mentioned UV-Ad-β-gal was intracutaneously administered at a concentration of 1×10^7 vp per mouse, and after 10 days, for tumor vaccination, irradiated ovarian cancer cells OVHM were subcutaneously transplanted at a concentration of 1×10^6 cells. Simultaneously, squamous ep. cancer cells SCC7 or ovarian cancer cells OVHM were subcutaneously transplanted at a concentration of 1×10^6 cells. Then, AdE3-1A1.3B infected A549 cells were administered six times at a concentration of 5×10^6 cells per mouse into a formed 5 - 10 mm tumor (in the Figure, shown by "OVHM-RT+Ad-β-gal→SCC7" and "OVHM-RT+Ad-β-gal→OVHM"). The treated mice showed a marked inhibition of tumor growth and proliferation in comparison to the control group. In the Figure, "SCC7" shows SCC7 tumor treated by the carrier cell without tumor vaccination, and "OVHM" shows OVHM tumor treated by the carrier cell without tumor vaccination. Especially, the tumor vaccination with irradiated OVHM followed by the carrier cell treatment, showed the formed OVHM tumor completely disappeared in all mice without recurrence.

Figure 23 shows the tumor vaccination with non-small-cell lung cancer A549 cells. In this experiment, irradiated A549 cells infected with adenovirus AdE3-1A1.3B at a concentration of 100 vp/cell were subcutaneously transplanted at a concentration of 1×10^6 cells per mouse. After 40 days, ovarian cancer cells OVHM were subcutaneously transplanted at a concentration of 1×10^6 cells. The mice (in the Figure, "AdE3-1.3B-infected A549→OVHM") also showed marked inhibition of tumor growth and proliferation in comparison to the control group (in the Figure, "OVHM" shows OVHM tumor treated by the carrier cell without tumor vaccination) with marked improvement in survival rate.

The above mentioned results show that the antitumor effect can be obtained even by tumor vaccination with different kinds of cancer cells.

Then, the effect of administering atelocollagen together with the carrier cell was investigated. In this experiment, A549 cells infected with a predetermined amount of adenovirus AdE3-1A1.3B were administered to 5- to 10-week-old (C57BL/6×C3/He) F1 mice at a concentration of 5×10^6 cells with atelocollagen at a final concentration of 0.1%. It was investigated whether the treatment decreased the death rate caused by the side effect due to the administering the adenovirus. The results are shown in Figure 24. In the Figure, the right bar shows the results of administering atelocollagen together with adenovirus AdE3-1.3B infected A549 cells at a concentration of 50 vp/cell or 250 vp/cell. The left and central bars show the results of administering adenovirus AdE3-1.3B infected A549 cells at a concentration of 5 vp/cell and 50 vp/cell, respectively (no atelocollagen is mixed). As shown in the Figure, simultaneously administering atelocollagen dramatically reduced the death rate caused by the side effects and the administration dose can be increased. This may be caused by the inhibition of adenovirus dispersion and the blockage of anti-adenovirus neutralizing antibodies by the atelocollagen.

As shown above, simultaneously administering atelocollagen and carrier cells suppressed the side effect and a high dose administration of adenovirus became possible.

Next, the adenovirus Ad-β-gal without inactivation treatment was administered once, twice or 3 times into a mouse to increase the anti-adenovirus antibodies in the blood by the booster effect. After that, the antitumor effect was investigated in each mouse. In this experiment, adenovirus Ad-β-gal was administered once, twice or 3 times to 5-week-old (C57BL/6×C3/He) F1 mice (administration at every four weeks at a concentration of 1×10^{10} vp),

thereafter, ovarian cancer cells OVHM were subcutaneously transplanted at a concentration of 1×10^6 cells per mouse to form 5 - 10 mm tumor and irradiated carrier cells were administered into the tumor. A549 cells, optionally mixed with 293 cells, were used as the carrier cells.

In case of mixtures of A549 cells and 293 cells, A549 cells infected with adenovirus AdE3-1A1.3B at a concentration of 50 vp/cell were administered at a concentration of 3.75×10^6 cells, and 293 cells infected with adenovirus AdE3-1A1.3B at a concentration of 10 vp/cell were administered at a concentration of 3.75×10^6 cells, respectively, into the tumor at each administration. These two kinds of carrier cells were administered six times in total. The results are shown in Figures 25 (a) and (b). In the case of administering only A549 cells, A549 cells infected with adenovirus AdE3-1A1.3B at a concentration of 50 vp/cell were administered at a concentration of 7.5×10^6 cells per mouse into the tumor at each administration. The carrier cells were administered six times in total. The results are shown in Figures 26 (a) and (b). In Figure 25 and Figure 26, "×1", "×2" and "×3" show the results of administering adenovirus Ad-β-gal once, twice and 3 times, respectively. As shown in these figures, the carrier cells showed antitumor effect even in anti-adenovirus antibody positive mice, caused by the several administrations of adenovirus Ad-β-gal. The mixture of A549 cells and 293 cells used as the carrier cells showed superior results to administering only A549 cells.

Figures 27 (a) and (b) show the *in vivo* antitumor effect by administering carrier cells (A549 cells) infected with adenovirus AdE3-1A1.3B and GM-CSF expression vector, and further together with atelocollagen. In this experiment, adenovirus Ad-β-gal was administered once, twice or 3 times (every four weeks at a concentration of 1×10^{10} vp) into 5-week-old (C57BL/6×C3/H3) F1 mice in a similar manner with the experiment shown in Figure 26.

Then, ovarian cancer cells OVHM were subcutaneously transplanted at a concentration of 1×10^{10} cells per mouse. After formation of a 5 - 10 mm diameter tumor, irradiated carrier cells (A549 cells) were administered into the tumor. The carrier cells were infected with adenovirus AdE3-1A1.3B at a concentration of 50 vp/cell and a GM-CSF expression vector (a vector with an inserted GM-CSF gene at the adenovirus E1 gene deficient site and downstream of the CMV promoter) at a concentration of 10 vp/cell. The prepared irradiated A549 cells were administered into the tumor at a concentration of 7.5×10^6 cells, together with atelocollagen (concentration at 0.1%), for each administration. These were administered three times in total.

In Figures 27 (a) and (b), "Ad-β-gal→AdE3-1A1.3B+GMCSF" shows the results of the above mentioned experiment, "Ad-β-gal→AdE3-1A1.3B" shows the results of six administrations of carrier cells (A549 cells) infected with adenovirus AdE3-1A1.3B at a concentration of 50 vp/cell into the tumor at a concentration of 7.5×10^6 cells for each administration. As shown in the Figure, three administrations of "AdE3-1A1.3B+GMCSF" showed a more potent *in vivo* antitumor effect than six administrations of "AdE3-1A1.3B", in all administrations of once, twice and 3 times of adenovirus Ad-β-gal.

The above results showed that infection of the oncolytic adenovirus and GM-CSF expression vector to the carrier cells was very effective in cancer therapy.

Figures 28 (a) and (b) show the effect of intraperitoneal administration of an iron preparation at the time of carrier cell administration. In this experiment, adenovirus Ad-β-gal was administered once, twice or 3 times (every four weeks at a concentration of 1×10^{10} vp for each administration) to 5-week-old (C57BL/6×C3/He) F1 mice. Then ovarian cancer cells OVHM were subcutaneously transplanted at a concentration of 1×10^6 cells per mouse. After formation of a 5 - 10 mm diameter tumor, irradiated carrier cells were administered into the tumor. A549 cells infected with adenovirus AdE3-1A1.3B at a concentration of 50 vp/cell were used as the carrier cells and administered at a concentration of 7.5×10^6 cells for each administration. At the time of administering the carrier cell, 0.01 mg of iron dextran (Fe-Dextran) was intraperitoneally administered as an iron preparation. These were administered three times (×3) in total (the iron preparation was also administered at every occasion).

In Figures 28 (a) and (b), "Ad-β-gal→AdE3-1A1.3B+Fe" shows the results of the above experiment. "Ad-β-gal→AdE3-1A1.3B" shows the results of six (×6) administrations of carrier cells (A549 cells) infected with adenovirus AdE3-1A1.3B at a concentration of 50 vp/cell in the tumor at a concentration of 7.5×10^6 cells for each administration. As shown in the Figure, three administrations of "AdE3-1A1.3B+Fe" showed a more potent *in vivo* antitumor effect than six administrations of "AdE3-1A1.3B" (in which case, only the carrier cells were administered), in all 1, 2 and 3 administrations of adenovirus Ad-β-gal.

The above results showed that a combined administering the carrier cell with an iron preparation is very effective in cancer therapy.

Then, the radiation dose in the radiation exposure treatment to the carrier cells before administration was investigated. In the experiment, 5-week-old nude mice were used and A549 cells were irradiated at different doses and then subcutaneously transplanted at a concentration of 1×10^7 cells per mouse, and the formation and growth of the tumor was observed. The results are shown in Figure 29. As shown in the Figure, formation and growth of the tumor was inhibited by setting the radiation dose at 120 Gy or over.

In an experiment using (C57BL/6×C3/He) F1 mice, Figure 30 shows the antitumor effect when the carrier cells (A549 cells) were treated by radiation with various doses. In this experiment, adenovirus UV-Ad-β-gal was administered to 5-week-old (C57BL/6×C3/He) F1 mice at a concentration of 1×10^{10} vp. After five weeks, the ovarian cancer cells OVHM were subcutaneously transplanted at a concentration of 1×10^6 per mouse. After formation of a 5 - 10 mm diameter tumor, carrier cells irradiated at a dose of 50 Gy, 100 Gy, 200 Gy or 400 Gy were administered into the tumor. A549 cells infected with adenovirus AdE3-1A1.3B at a concentration of 50 vp/cell were used as the carrier cells and administered at a concentration of 7.5×10^6 cells for each administration. The carrier cells were administered six times in total. The results showed a more favorable result at a radiation dose of 400 Gy than at a radiation dose of 200 Gy.

Figure 31 shows the amount of infection of the oncolytic virus to the carrier cell. In this experiment, adenovirus Ad-β-gal was administered to 5-week-old (C57L/6×C3/He) F1 mouse at a concentration of 1×10^{10} vp. After four weeks, ovarian cancer cells OVHM were subcutaneously transplanted at a concentration of 1×10^6 cells. After formation of a 5 - 10 mm diameter tumor, carrier cells (A549 cells) irradiated at a dose of 250 Gy were administered into the tumor. The amount of infection of adenovirus AdE3-1A1.3B to the carrier cell was set at 100 vp/cell, 250 vp/cell or 500 vp/cell. The carrier cells were administered at a concentration of 7.5×10^6 cells for each administration. Together with the carrier cell administration, atelocollagen (concentration 0.1%) was concurrently administered in the tumor. These were administered six times in total. The results showed most favorable result at the infection concentration of 250 vp/cell, and found that concentrations of 150 - 400 vp/cell gave favorable results.

Figures 32 (a) and (b) show the effect of tumor vaccination in a similar experiment to that shown in Figure 31. In this experiment, adenovirus Ad-β-gal was administered to 5-week-old

(C57L/6×C3/He) F1 mice at a concentration of 1×10^{10} vp. After four weeks, for tumor vaccination, ovarian cancer cells OVHM-RT irradiated at a dose of 80 Gy were subcutaneously transplanted at a concentration of 1×10^{6} cells. Then, ovarian cancer cells OVHM were subcutaneously transplanted at a concentration of 1×10^{6} cells. After formation of a 5 - 10 mm diameter tumor, carrier cells treated irradiated at a dose of 250 Gy were administered into the tumor. A549 cells infected with adenovirus AdE3-1A1.3B at a concentration of 50 vp/cell were used as the carrier cells and administered at a concentration of 7.5×10^{6} cells for each administration. Together with the carrier cell administration, atelocollagen (concentration 0.1%) was concurrently administered in the tumor. These were administered three times in total. The results showed marked inhibition of tumor growth and proliferation in the mouse with tumor vaccination (in the Figure, "OVHM-RT→A549") in comparison to the mouse without tumor vaccination (in the Figure, "A549"). The survival rate was also greatly improved.

The above results show that the use of tumor vaccination provides a favorable antitumor effect together with the use of the cancer gene therapeutic drug of the present disclosure.

Industrial applicability

As described above, the cancer gene therapeutic drug of the present disclosure can be applied to almost all malignant tumors and can be expected to exhibit potent antitumor effect including ovarian cancer, squamous epithelium cancers (e.g. uterine cervix cancer, cutaneous carcinoma, head and neck cancer, esophageal cancer and lung cancer), digestive tract cancers (e.g. colon cancer, pancreatic cancer, hepatic cancer and gastric cancer), neuroblastoma, cerebral tumor, mammary cancer, testicular cancer and prostatic cancer.